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Electrophoretic and Morphological Confirmation of Interspecific Hybridization between *Polystichum kruckebergii* and *P. munitum*

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The genus *Polystichum* (Dryopteridaceae) comprises from 160 (Tryon & Tryon, 1982) to 175 (Copeland, 1947) species and is nearly worldwide in distribution. Substantial morphological diversity exists within *Polystichum* (Barrington, 1985), and considerable taxonomic confusion has characterized *Polystichum* since its description in 1799. Systematic problems in *Polystichum* stem largely from extensive hybridization and allopolyploidy (Knobloch, 1976; Tryon & Tryon, 1982; Mickel in Barrington, 1985; D. Wagner in Barrington, 1985; W. Wagner in Barrington, 1985), which tend to obscure species boundaries and make morphological comparisons difficult. Furthermore, the production of interspecific sterile hybrids has created additional taxonomic confusion (Manton, 1950; Nakaike, 1973; W. Wagner, 1973; Daigobo, 1974; D. Wagner, 1979).

A prime example of the reticulate evolution typical of the genus (D. Wagner, 1979, in Barrington, 1985; W. Wagner in Barrington, 1985) is found in the *Polystichum* complex from western North America. This group consists of five diploid species, three once-pinnate (*P. imbricans*, *P. lonchitis*, and *P. munitum*) and two highly dissected (*P. dudleyi* and *P. lemmonii*). There are five tetraploids, *P. andersonii*, *P. braunii*, *P. californicum*, *P. kruckebergii*, and *P. scopulinum*; all but *P. braunii* incorporate various combinations of the diploid genomes discussed above (W. Wagner, 1973; D. Wagner, 1979). All of these species are restricted to western North America except *P. braunii* and *P. lonchitis*, which are circumboreal in distribution, and *P. scopulinum*, which also occurs sporadically in eastern North America. [Following D. Wagner (1979), *P. lemmonii* is considered distinct from the South American *P. mohrioides*.] *Polystichum setigerum*, reported to be a sexual hexaploid derived from *P. munitum* and *P. braunii* (D. Wagner, 1979), is also a member of this complex.

Several sterile hybrids among these species have been reported from North America, including *P. californicum* × *munitum* (Callan, 1972; W. Wagner, 1973), *P. braunii* × *munitum* (Alverson, unpubl.), *P. andersonii* × *munitum*, *P. californicum* × *dudleyi*, *P. dudleyi* × *munitum*, *P. lemmonii* × *munitum*, *P. lemmonii* × *scopulinum*, and *P. munitum* × *scopulinum* (W. Wagner, 1973), and *P. braunii* × *lonchitis* in Europe (Sleep & Reichstein, 1967). In addition, *P. braunii* has hybridized with the eastern North American *P. acrostichoides* (Thompson & Coffin, 1940; Barrington, 1986), and hybrids between *P. lonchitis* and *P. ac-*

rostichoides have also been reported (W. Wagner & Hagenah, 1954). Furthermore, both *P. braunii* and *P. lonchitis* have hybridized with the European species *P. aculeatum* and *P. setiferum* (reviewed by Sleep & Reichstein, 1967). In this paper we provide morphological and electrophoretic evidence for hybridization between *P. munitum*, a diploid, and *P. kruckebergii*, an allotetraploid whose presumed diploid progenitors are *P. lemmonii* and *P. lonchitis* (W. Wagner, 1973).

The putative hybrid plants under study are readily recognized as unusual; they possess moderately incised pinnae, which in nature are twisted relative to the axis of the rachis. The plants are medium-sized, with leaf blades of mature plants ranging from 35–42 cm by 5–6 cm; the plants are generally larger than the small, rock-dwelling polystichums, such as *P. kruckebergii*, but smaller than the large, forest-dwelling taxa, such as *P. munitum*. Close examination of the sori revealed abortive sporangia, as well as irregularly sized and shaped spores; many of the spores were shriveled and presumably abortive. Thus, the plants displayed features characteristic of interspecific hybrids (W. Wagner, 1968).

The putative hybrid between *P. munitum* and *P. kruckebergii* originated under an unusual set of geological circumstances. Typically, *P. munitum* and *P. kruckebergii* are not sympatric; *P. munitum* usually inhabits moist woods at lower elevations, and *P. kruckebergii* usually occurs at higher elevations, often on ultramafic substrates. The putative hybrids occur with *P. kruckebergii* and *P. munitum* at an elevation of approximately 1065 m on the lower slopes of Devil's Thumb in the Coal Creek drainage on the west side of the Cascades in Snohomish County, Washington. The plants grow at the base of a large glacial erratic of peridotite (an ultramafic mineral) that originated higher up on the mountain slope. This population of *P. kruckebergii* and the putative hybrids occur in an *Abies amabilis-Tsuga heterophylla* forest, at a much lower elevation than is typical of *P. kruckebergii*. This habitat and elevation are more typical of *P. munitum* in this region. *Polystichum lonchitis* was also present on a non-ultramafic outcrop 100 m away from the hybrid site and, instead of *P. munitum*, could conceivably be the once-pinnate parent of the putative hybrids. *Polystichum lonchitis* was therefore included in this study.

The origin of an interspecific hybrid can be extremely difficult to determine with certainty when only morphological criteria are employed, particularly in a plant group known for its phenotypic plasticity and environmentally induced variability (Barrington, 1985). Therefore, we utilized genetic markers in addition to morphological analyses to determine the origin of the putative hybrids.

Materials and Methods

Morphological data were obtained from pressed fronds of *P. munitum*, *P. kruckebergii*, and the putative hybrids collected at the hybrid locality. Vouchers were deposited at WS.

We electrophoretically examined the five putative hybrid plants, five individuals of *P. munitum*, and four individuals each of *P. kruckebergii* and *P. lonchitis* from the hybrid locality. Leaf material was collected in the field and stored in

plastic bags with wet paper towels under refrigeration until electrophoresis was conducted.

Electrophoretic procedures generally followed those of D. Soltis et al. (1983). Leaf tissue was prepared using the tris-HCl grinding buffer-PVP solution of D. Soltis et al. (1983); three grams of PVP were used per 25 ml of grinding buffer. Starch gel concentration was 12.5%.

The following enzymes were examined: aspartate aminotransferase (AAT), fluorescent esterase (FE), leucine aminopeptidase (LAP), malate dehydrogenase (MDH), phosphoglucoisomerase (PGI), phosphoglucomutase (PGM), shikimate dehydrogenase (SkDH), and triosephosphate isomerase (TPI). AAT, FE, LAP, PGI, and TPI were resolved on a modification of gel and electrode buffer system 8 of Soltis et al. (1983); the gel buffer was composed of 0.033 M tris, 0.005 M citric acid, 0.004 M lithium hydroxide, 0.030 M boric acid, pH 7.6, and the electrode buffer was composed of 0.039 M lithium hydroxide, 0.263 M boric acid, pH 8.0. MDH, PGM, and SkDH were resolved on gel and electrode buffer system 9 of D. Soltis et al. (1983). Staining for all enzymes followed D. Soltis et al. (1983), except LAP, which followed Soltis and Rieseberg (1986).

The genetic control of electrophoretic banding patterns was readily interpreted based on the known subunit structure and subcellular localization of the enzymes (Gottlieb, 1981, 1982). Loci were numbered sequentially, with the most anodally migrating locus designated *1*, the second most anodal locus *2*, and so on. Similarly, allozymes were denoted alphabetically, with the fastest migrating allozyme designated *a*, the second fastest allozyme *b*, and so on.

Results

Morphology.—Table 1 compares *P. kruckebergii*, *P. munitum*, and the putative hybrids for 11 distinguishing morphological features. In many cases, the putative hybrids were intermediate to the parental species, though the hybrid did not exhibit uniform intermediacy. For example, the ratio of pinna length to pinna width, reduction of lower pinnae to triangular segments, and the rotation of pinnae from the plane of the rachis were all closer to the condition of *P. kruckebergii*. In contrast, the rather slight degree of pinna dissection, the frond texture, and the marginal teeth of the pinnae were all more typical of *P. munitum*. The non-costal infralaminar scales are noteworthy in that scales representing both of the parental phenotypes, as well as intermediate conditions, could be found on a single pinna.

Spores are one feature in which hybrid intermediacy was not consistently expressed. *Polystichum munitum* has small, yellowish, translucent spores that average 33 μm in length. Spores of the tetraploid *P. kruckebergii* are much larger, averaging 42 μm in length; they are dark brown in color and are only slightly translucent. Spores of both species are distinctly monolete and relatively uniform in size. In contrast, spores of the hybrid are generally spherical, when not shriveled and malformed. It is noteworthy, however, that many of the spores taken from the hybrid plants were large and well formed. These large, spherical spores

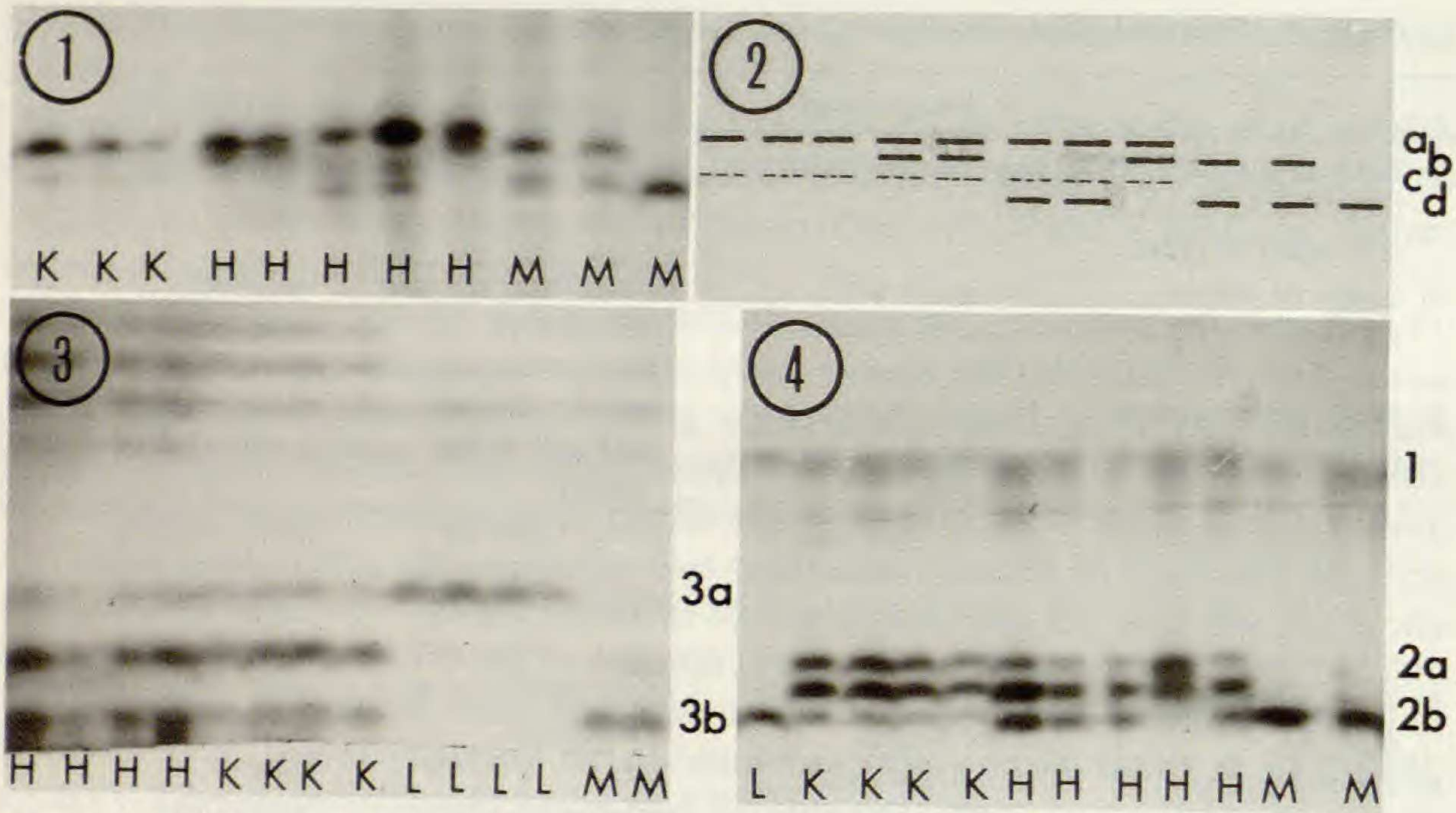
TABLE 1. Morphological Characteristics of *P. kruckebergii*, *P. munitum*, and Their Hybrid.

	P. kruckebergii	hybrid	*P. munitum*
1) Ratio of pinna length to its width at mid-point of pinna	approximately 2:1	approximately 3:1	approximately 6:1
2) Shape of lowest pinnae	triangular with nearly equilateral sides	triangular to ovate-lanceolate	lanceolate
3) Pinna orientation in nature	rotated relative to the axis of the rachis	rotated relative to the axis of the rachis	plane relative to the axis of the rachis
4) Dissection of pinnae	pinnae fully pinnatifid, at least the first 1–3 lobes	pinnae dissected ⅓–⅔ way to the costa	pinna margins toothed, sometimes incised, but not dissected
5) Teeth of the pinna margins	teeth tending to be spreading	teeth tending to be incurved	teeth mostly strongly incurved
6) Frond texture	somewhat soft	firm	firm
7) Indusium margin	more or less entire	erose, with occasional cilia	long ciliate
8) Non-costal infralaminar scales	nearly linear, mostly with few, short basal projections	variable; ranging from short with many basal projections to lanceolate or linear-lanceolate, with few or no basal projections	lanceolate, with an arachnoid base, the basal projections often as long as the body of the scale
9) Spore shape	monolete, uniform	spherical, or shrunken and irregular, often variable in size and outline	monolete, uniform
10) Spore color	dark brown, somewhat translucent	dark brown, opaque	yellowish-brown, translucent
11) Mean exospore length (S.D.)	43 μm (5 μm)	42 μm (8 μm)	33 μm (2 μm)

may be unreduced "mitospores" similar to those described by Morzenti (1962). Morphological characteristics of the putative hybrids and the circumstances under which the hybrids occur clearly suggest that these plants originated by hybridization between *P. kruckebergii* and *P. munitum*.

Electrophoresis.—Eleven loci were interpreted: *Aat*, *Fe-1*, *Lap*, *Mdh-1*, *Pgi-2*, *Pgm-1*, *Pgm-2*, *Skdh*, *Tpi-1*, *Tpi-2*, and *Tpi-3*. The observed enzyme bands migrated anodally for all enzymes.

To document hybridization, electrophoretic investigations of hybridization require genetic markers differentiating the putative parental species. The three possible parental species, *P. munitum*, *P. kruckebergii*, and *P. lonchitis*, possess different genotypes for *Fe-1*, *Lap*, *Pgm-1*, *Pgm-2*, and *Tpi-3* (Figs. 1–3). At *Lap*,



FIGS. 1–4. Starch gels in *Polystichum*. In all figures, numbers and letters in margins designate loci and alleles, respectively. K = *Polystichum kruckebergii*; M = *P. munitum*; L = *P. lonchitis*; H = hybrid. 1. LAP. 2. Interpretive drawing of Fig. 1. 3. TPI. 4. PGI.

all individuals of *P. kruckebergii* possessed the fixed heterozygous genotype *Lap-ac* (Figs. 1, 2). *Polystichum lonchitis* exhibited only allele *Lap-c*, and plants of *P. munitum* displayed *Lap-b*, *Lap-d*, or both of these alleles (Figs. 1, 2). The five putative hybrids possessed one of the following genotypes: *Lap-abc* (three individuals) or *Lap-acd* (two individuals), clearly combining the genotypes of *P. munitum* and *P. kruckebergii* (Figs. 1, 2).

At *Fe-1*, three of the putative hybrids possessed allele *Fe-1b*, the allele found in all *P. kruckebergii* individuals examined. Another putative hybrid possessed the genotype *Fe-1bc*, combining allele *b* of *P. kruckebergii* and allele *c* which was found in all individuals of *P. munitum* from the hybrid site. One putative hybrid did not display FE activity. All individuals of *P. lonchitis* exhibited allele *d*. Data from *Fe-1* therefore provide limited support for the hypothesis that the hybrids are derived from *P. kruckebergii* and *P. munitum*.

Although the putative hybrids possessed identical enzyme bands to those of *P. kruckebergii* at *Pgi-2*, the staining intensities of these bands were clearly different (Fig. 4). All individuals of the tetraploid *P. kruckebergii* exhibited the fixed heterozygous pattern typical of an allotetraploid, with balanced staining. However, dosage effects were observed in the putative hybrids. All of the putative hybrids displayed unbalanced heterozygosity at *Pgi-2* (Fig. 4). Four individuals possessed extra doses of allele *b*, the allele typical of *P. munitum* and *P. lonchitis*, and one individual had an additional dose of allele *a*, an allele present in the fixed heterozygous pattern of *P. kruckebergii* and observed in other populations of *P. munitum* (P. Soltis & D. Soltis, 1987). Unbalanced staining would be expected in a triploid hybrid between a diploid and an allotetraploid that share alleles.

The other loci examined did not provide information useful in determining

the parentage of the hybrids. *Polystichum kruckebergii* can be distinguished from *P. munitum* and *P. lonchitis* at *Tpi-1* and *Tpi-2*, but the latter two species cannot be differentiated (Fig. 3). The tetraploid *P. kruckebergii* exhibited fixed heterozygosity at *Tpi-3* (Fig. 3). In addition, a five-banded pattern was observed in the more anodal zone of activity for TPI (Fig. 3). However, it is unknown whether this represents fixed heterozygosity at *Tpi-1* or *Tpi-2* or both of these loci. The putative hybrid could not be distinguished from *P. kruckebergii* at any of the TPI loci (Fig. 3).

The loci *Aat*, *Mdh-1*, and *Skdh* were monomorphic for all plants examined. Although the three possible progenitors were differentiated at *Pgm-1* and *Pgm-2*, the banding patterns were not clearly resolved in the putative hybrids.

Discussion

The morphological and allozymic data presented herein confirm the occurrence of hybridization between *Polystichum munitum* and *P. kruckebergii*. The putative hybrids are morphologically intermediate between the proposed parental species for several characters. Furthermore, the hybrids clearly combine the alleles of *P. munitum* and *P. kruckebergii* at *Lap*. The data obtained for all other loci examined are also consistent with this hypothesis of hybridization. The data for *Lap* and *Fe-1* rule out the possibility that *P. lonchitis* is one of the parental species.

In the five hybrid individuals examined we detected three different genotypes across all loci. This indicates that hybridization between different *P. munitum* and *P. kruckebergii* individuals occurred at least three times in this mixed population; the several hybrid individuals were not the result of vegetative reproduction following a single hybridization event.

Because the tetraploid *P. kruckebergii* is thought to have *P. lemmonii* and *P. lonchitis* as its diploid progenitors (W. Wagner, 1973; D. Wagner, 1979), the genomic composition of the hybrids presumably comprises three distinct genomes: *P. munitum*, *P. lonchitis*, and *P. lemmonii*. This combination could also result from a hybrid between *P. lonchitis* and *P. scopulinum*, provided that at least some populations of *P. scopulinum* have the parentage *P. lemmonii* × *munitum* s. s., as proposed by W. Wagner (1973). More recently, however, D. Wagner (1979) suggested that the once-pinnate diploid progenitor of *P. scopulinum* was *P. imbricans* rather than *P. munitum*. Further allozymic investigations should provide additional information regarding evolutionary relationships in this species complex.

The detection of yet another interspecific hybrid in *Polystichum* is not surprising, particularly given the widespread occurrence of hybridization within the genus (Knobloch, 1976; Tryon & Tryon, 1982; Mickel in Barrington, 1985; W. Wagner in Barrington, 1985). It is noteworthy that five of the seven interspecific *Polystichum* hybrids previously reported from western North America involve *P. munitum* as a parental species. This may be due to the more widespread distribution of this species, relative to other western North American *Polystichum* species. However, the frequency with which *P. munitum* hybridizes with other

species may also reflect the breeding system of this species. *Polystichum munitum* is almost completely outcrossing; intragametophytic selfing estimates in populations of *P. munitum* ranged from 0 to 3% (P. Soltis & D. Soltis, 1987). This outcrossing breeding system provides the potential for interspecific hybridization whenever *P. munitum* occurs in the same locality as other *Polystichum* species. Other *Polystichum* species are also outcrossing. For example, in *P. imbricans* intragametophytic selfing estimates ranged from 0 to 17% (D. Soltis and P. Soltis, 1987). Data regarding the mating systems of *P. kruckebergii* and other *Polystichum* species are not yet available; however, the frequency of hybridization in this genus suggests that high outcrossing rates may be typical of other species of *Polystichum*.

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REVIEW

"*Iconographia palynologica pteridophytorum Italiae,*" by E. Ferrarini, F. Ciampolini, R. E. G. Pichi Sermolli, and D. Marchetti. Webbia 40:1–202. 1968.

This collaborative work, in Italian, by three biologists at the University of Siena and R. E. G. Pichi Sermolli at the University of Perugia, has resulted in an impressive series of scanning electron micrographs of spores of the 65 species of Italian Pteridophyta. More than 500 SEM's, assembled in 71 plates, include 3–8 figures and surface details for each taxon. The conservative nature of spores is evident in the similar details of surface and elators of the nine species of *Equisetum.* Similarities of surface contours in many genera with monolete spores suggest there may be broad, general alliances between these genera.

An extensive glossary, illustrated by fine drawings, includes terms as nexine and sexine, not applicable to spores. The binary key to genera, based on spore morphology is not easily used. Differences of a few microns in the headings leading to *Phyllitis* and *Asplenium* or to species of *Polypodium* are difficult choices. The heading "Spore senze perina" leading to *Polypodium* unfortunately is an inaccuracy that undoubtedly persists from light microscope observations in which the perine was not evident. Scanning and transmission microscope work on spores show, with few exceptions, that spores of all Filicineae have perine, or perispore.

In addition to descriptions of the spores, the text consists of comments on nomenclature and the cytology of the species. In light of the emphasis on cytology it is disappointing to find the SEM magnifications are inconsistent. Differences in size of spores of *Asplenium ruta-muraria,* shown at × 1080 for the autotetraploid subsp. *ruta-muraria,* and at × 750 for the diploid subsp. *dolomitica,* cannot be readily visualized.

This atlas of spores will be particularly valued as a reference for comparison of surface morphology of these 65 species with that of spores from other regions.

The fine details depicted in SEM spore studies such as this require special attention to reproduction of the micrographs for publication. This unfortunately has contributed to the cost of this volume, which at 130,000 lira, is somewhat more than one hundred dollars.—ALICE F. TRYON, Harvard University Herbaria, Cambridge, MA 02138.